

TECHNICAL NOTE

## The Direct Determination of Urinary Oxalate by Non-Suppressed Ion Chromatography<sup>1)</sup>

Katy Pfeiffer<sup>1</sup>, Wolfgang Berg<sup>1</sup>, Daniel Bongartz<sup>2</sup> and Albrecht Hesse<sup>2</sup>

<sup>1</sup> Forschungslabor der Klinik und Poliklinik für Urologie (Direktor Prof. Dr. J. Schubert) am Klinikum der Friedrich-Schiller-Universität Jena, Jena, Germany

<sup>2</sup> Experimentelle Urologie, Klinik und Poliklinik für Urologie (Direktor Prof. Dr. S. C. Müller) der Rheinischen Friedrich-Wilhelms-Universität Bonn, Bonn, Germany

**Summary:** In this paper we describe a simple ion chromatographic method for determination of oxalate in urine. Acidified urine was diluted 1 : 2 with 0.03 mol/l benzidine hydrochloride in 0.3 mol/l boric acid to precipitate sulphate. The supernatant was passed through a C<sub>18</sub>-cartridge and 100 µl of eluant were injected into an ion chromatographic system. Oxalate was measured by non-suppressed conductivity detection. The detection limit for urinary oxalate was 0.05 mmol/l. The recovery for spiked urine samples was 101.5% with a CV of 4.5%. The within- and between-assay coefficients of variation were less than 4.5% and 2.5%, respectively. We found the results obtained by this method to be statistically equivalent to an enzymatic assay and a different ion-chromatographic method.

### Introduction

Urolithiasis is still a widespread disease. Almost 70% of the renal stones consist of two forms of calcium oxalate (1). In addition to the stone analysis, the reliable analysis of oxalate in body fluids, especially in urine, is of great importance for the diagnosis and adequate metaphylaxis of calcium oxalate urolithiasis. In the last thirty years many methods for the determination of urinary oxalate have been developed (2, 3). The most applied procedures are colorimetric methods (4, 5), fluorometry (6), atomic absorption spectrophotometry (7), gas chromatography (8, 9), isotope dilution (10, 11), enzymatic methods using oxalate oxidase<sup>2)</sup> (12, 13, 15) or oxalate decarboxylase<sup>2)</sup> (14, 15), isotachopheresis (8), and high-performance liquid chromatography (HPLC), especially ion chromatography (13, 16–22). In the last decade both the colorimetric method by *Hodgkinson & Williams* (4), which is inaccurate, insensitive, expensive and laborious, and the isotachopheretic method and gas chromatography have been replaced by specific methods like enzymatic procedures and HPLC-techniques.

The ion chromatographic techniques usually apply suppressed conductivity detection of the oxalate ion, although for the oxalate determination in urine, suppression is not required with respect to sensitivity. We describe a simple oxalate assay using ion chromatography with non-suppressed conductivity detection after removal of sulphate. The chromatographic technique, analytical conditions and its clinical application are reported in this paper.

### Materials and Methods

#### Equipment

The chromatographic system consisted of an HPLC-pump (WATERS 510, Waters Chromatography Division, USA), an autosampler (WATERS WISP 712) with variable sample loop, a non-suppressed conductivity detector (WATERS 431) and an analytical anion-exchange column (WATERS IC pak anion high capacity, 4.6 × 140 mm, 10 µm particle size). Chromatograms were recorded on an integrator (WATERS Data Module 746). For sample clean-up SepPak C<sub>18</sub>-plus cartridges (360 mg packing material, 55–105 µm particle size, Millipore Corp., USA) were used.

#### Reagents

Boric acid, sodium tetraborate decahydrate, glycerin, n-butanol, acetonitrile, hydrochloric acid (300 g/kg, about 8 mol/l), ammonium sulphate and ammonium oxalate were obtained from E. Merck (Darmstadt, Germany); sodium gluconate was purchased from Sigma (SIGMA Chemical Co., USA) and benzidine dihydrochloride from Fluka (Fluka Chemie AG Buchs, Switzerland). All reagents used were of analytical grade and all solvents were of HPLC-grade. The 0.03 mol/l benzidine dihydrochloride/0.3 mol/l boric acid solution was prepared daily. The stock solution of ammonium oxalate had a concentration of 0.025 mol/l and was storable up to one week. The standards were freshly prepared by dilution of the 25 mmol/l oxalate stock solution, addition of HCl (10 ml 8 mol/l per litre solution), and addition of 20 mmol/l sulphate. A 0.05 mol/l ammonium sulphate stock solution was prepared for precipitation experiments.

#### Preparation of urine samples

24-h urines were collected in polyethylene bottles containing 15 ml concentrated hydrochloric acid (300 g/kg) for preservation and to obtain pH values < 2.0. A 3 ml aliquot of the urine was diluted with 3 ml of a 0.03 mol/l benzidine dihydrochloride solution (CAUTION: highly carcinogenic) in 0.3 mol/l boric acid, agitated, and filtrated. A reversed phase C<sub>18</sub>-cartridge was activated by passing 2 ml methanol and 4 ml aqua dest. The first millilitre of the urine sample was rejected and the following 3 ml were collected in a 4 ml vial.

#### Chromatographic conditions

The mobile phase was a borate-gluconate buffer containing 1.47 mmol/l sodium gluconate, 5.82 mmol/l boric acid, 1.31 mmol/l sodium tetraborate decahydrate, 5 ml/l glycerol, 120 ml/l acetonitrile and 20 ml/l n-butanol. The eluant had a pH value of 8.5 and its conductivity was 275 µS. The flow rate was 2 ml/min. The elu-

<sup>1)</sup> Funding organisation

This work is supported by the Deutsche Forschungsgemeinschaft.

<sup>2)</sup> Enzymes

Oxalate decarboxylase, EC 4.1.1.2

Oxalate oxidase, EC 1.2.3.4

ant was degassed by vacuum filtration through a membrane filter (0.5  $\mu\text{m}$ , Millipore Corporation, USA). The detector sensitivity was set to 0.1 S full scale. Pretreated sample material (100  $\mu\text{l}$ ) was injected onto the column. The retention time of oxalate was 27 minutes at a constant column temperature of 26  $^{\circ}\text{C}$ . Oxalate was quantified via peak area and external standards.

The separation column gave reproducible retention times up to 650 injections. After that period a regeneration with 100 ml of twofold concentrated eluant with a low flow rate < 1.0 ml/min was necessary. After approximately 800 injections the column should be replaced.

## Results and Discussion

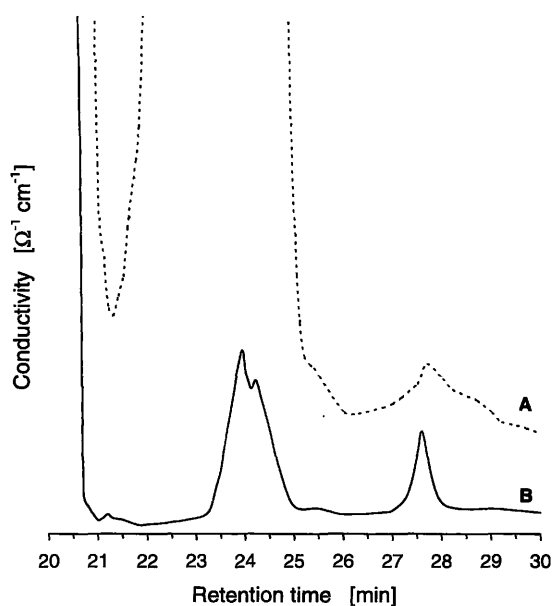
The acidification of the urine samples during collection is often applied. First of all it prevents oxalate precipitation. Second it delays the formation of oxalate from ascorbate (13, 26). Furthermore, combined with storage at  $-18^{\circ}\text{C}$  it is a way of preservation (25).

The interaction of the oxalate peak with the sulphate peak is the main problem with all anion exchange columns currently available. This interaction increases the determination limit and disturbs the accurate evaluation of the oxalate peak area. Experiments to obtain a better separation of sulphate and oxalate by variation of the strength of the eluting solvent, the flow rate or the temperature of the column gave unsatisfying results. Series of measurements applying solutions with 0.2 mmol/l of oxalate and varying sulphate concentrations from 5–35 mmol/l were performed. These measurements showed that 20 mmol/l of sulphate results in deviated oxalate values. Therefore, it was necessary to eliminate the sulphate.

The first attempts of sulfate precipitation with barium chloride as suggested by Toyoda (22) were not successful because of a simultaneous precipitation of oxalate in the above mentioned concentration range. Another insoluble sulphate salt is benzidine sulphate (24). A comparable insoluble oxalate compound should not be formed under the applied reaction conditions. To investigate this fact aqueous solutions and urines were treated with aqueous benzidine dihydrochloride solutions with different concentrations to find the optimal concentration for the quantitative precipitation of sulphate. We assumed an average physiological concentration of 20 mmol/l urinary sulphate. The results of these experiments confirmed that the oxalate is not influenced if urines are diluted with benzidine dihydrochloride solutions with a concentration of 30 mmol/l in the ratio 1 : 1. To examine the dependency of sulphate precipitation on the pH value we tested urines with pH values ranging from 1–8. We found that pH values ranging from 1–3 were optimal for the sulphate precipitation and oxalate recovery. A typical chromatogram before and after precipitation of sulphate is shown in figure 1.

For the prevention of the oxalate formation from ascorbate by alkaline pH values during the measurements the urines were diluted with 0.3 mol/l boric acid solution as suggested (13, 26). Boric acid is part of the mobile phase and the pH value of the eluant used was 8.5 in comparison to pH 10 of the widely used carbonate/hydrogencarbonate buffer (13, 17). The steps of dilution with boric acid and the addition of benzidine dihydrochloride were combined by dissolving the benzidine dihydrochloride in 0.3 mol/l boric acid solution.

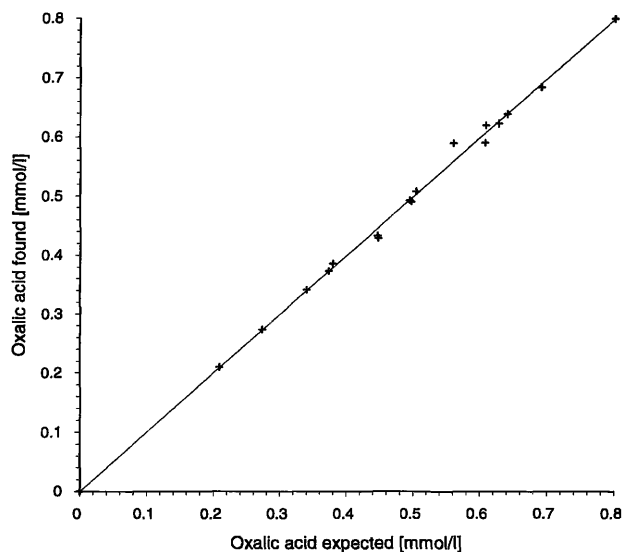
Other interfering phenomena with ions occurring in urine were not found. This was tested by adding halogenides, phosphates, ascorbate, glyoxylic acid, glycolic acid, and citric acid to acidified standard solutions in physiological concentrations (23). The calibration curve for the quantitative determination of oxalate was established by using aqueous standard solutions with concentrations ranging from 0.05–1.0 mmol/l. The standards were pretreated and analyzed analogously to the procedure described for urine samples. The peak area increases linear with the oxalate concentration in the range of from 0.05 mmol/l to 1.0 mmol/l. This range is sufficient, since 98% of all urine samples are found in this region. The detection limit for urinary oxalate was 0.05 mmol/l.



**Fig. 1** Typical chromatogram of a 24 h urine containing 0.18 mmol/l oxalate (A: before separation of sulphate, B: after separation of sulphate).

To characterize the analytical conditions of the method we evaluated the recovery of oxalate added and the within-run and between-batch precision. To determine the recovery we measured the urines before and after spiking with oxalate. Oxalate was added to four urines to obtain an additional oxalate concentration of 0.1, 0.2, 0.3, 0.4, and 0.5 mmol/l, respectively. All 20 samples were measured twice. In the range from 0.1 to 1.0 mmol/l oxalate we found a mean recovery of 101.5% with a CV of 4.5%. Figure 2 shows the recovery results by comparison of expected and measured oxalate concentration.

For the investigations of between-batch precision three urines with low, middle and high oxalate concentrations were measured twice a day over the course of 10 days. Additionally, the oxalate concentration of the same urine sample was determined twenty times to gain information about within-run precision. The means of oxalate concentration and the CV are summarized in table 1. These investigations delivered accurate results with oxalate concentrations ranging from 0.1 mmol/l to 1.0 mmol/l. The CV was best at high and



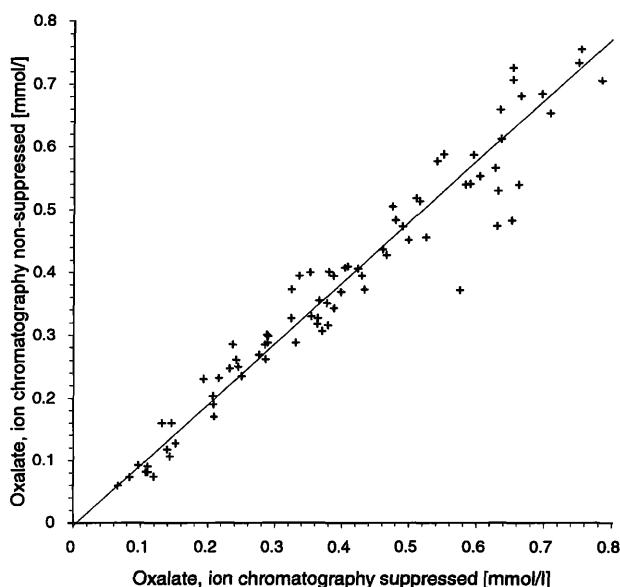
**Fig. 2** Recovery of the method (solid line = linear regression,  $y = 0.9992x - 0.0003$ ).

**Tab. 1** Within-run and between-batch imprecision of the method (three urine samples with low (A), middle (B), and high (C) oxalate concentration).

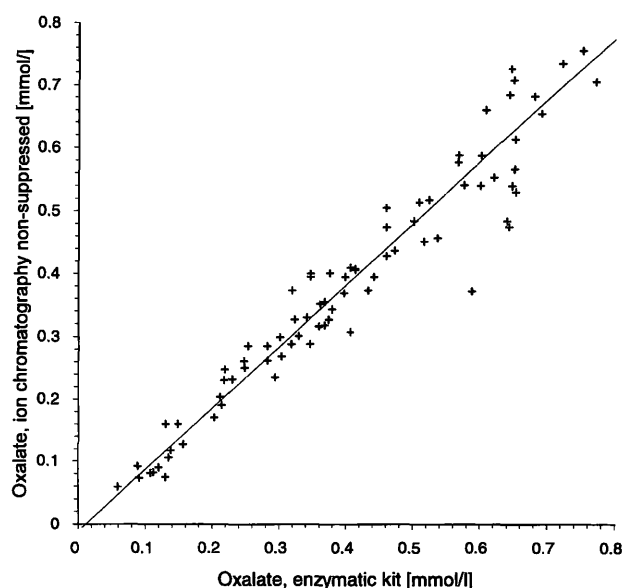
Sample	Within-run			Between-batch		
	A	B	C	A	B	C
Mean (mmol/l)	0.109	0.455	0.729	0.108	0.454	0.702
Minimum (mmol/l)	0.096	0.402	0.692	0.100	0.438	0.685
Maximum (mmol/l)	0.137	0.493	0.779	0.116	0.467	0.720
Standard deviation (mmol/l)	0.008	0.019	0.016	0.006	0.009	0.011
CV (%)	7.3	4.2	2.2	5.6	2.0	1.6

middle oxalate concentrations. At the concentration of 0.1 mmol/l the CV was worst but still satisfying.

According to *Passing & Bablok* (27, 28) we compared the method described, a different ion chromatographic method with a more basic eluant and chemical background suppression (13), and a commercial enzymatic kit (Sigma Chemical Co., product No. 591) using oxalate oxidase (13). This was performed by measuring 80 different urines from healthy subjects and calcium oxalate stone formers (Department of Urology, University of Bonn) with each of the methods. Figures 3 and 4 show the correlation between the three methods compared (regression parameter enzymatic kit – unsuppressed ion chromatography). The comparison of methods



**Fig. 3** Correlation of the new non-suppressed ion chromatographic method and the suppressed ion chromatographic method ( $n = 80$ ; solid line: regression according to *Passing & Bablok*,  $y = 0.971x - 0.005$ ).



**Fig. 4** Correlation of the new ion chromatographic method and an enzymatic oxalate kit ( $n = 80$ ; solid line: regression according to *Passing & Bablok*,  $y = 0.983x - 0.012$ ).

showed statistically identical results for all three methods. Because of the convenience of the method described, it is possible to measure up to 40 samples per day.

The calculation of cost depends on the amount of samples that are to be analyzed. The estimated sample-independent charge for the enzymatic method is about 7 US\$ (material, working time, overhead). Not included in this calculation is the purchase of a photometer, because it is available in most laboratories. The estimated charges for a suppressed and a non-suppressed ion chromatographic analysis of oxalate (including purchase, maintenance, and depreciation of the chromatographic system) are about 5 US\$ and 4 US\$. The calculations for the chromatographic system are based on a throughput of about 3500 samples/year for 5 years. The higher expenses for the suppressed system are due to the reduced variety of suppressor devices and/or suppressed chromatographic systems.

## Conclusions

The HPLC-method for the determination of urinary oxalate is accurate. The results with regard to quality were excellent and the method is simple to perform. All analytical tests gave accurate results and the method is very reliable. A comparison with two different well-established procedures showed good correlations. The problem of interfering sulphate was solved by using a new and uncomplicated procedure of almost quantitative precipitation of sulphate using benzidine dihydrochloride. An advantage of the employed system over other ion chromatographic methods is the use of a borate buffer as mobile phase with pH value of 8.5. This reduces the risk of oxalogenesis from ascorbate. Furthermore, the method can be adapted to every existing HPLC system.

## Acknowledgements

We would like to thank Dr. S. Albrecht (University of Dresden) for helpful discussions.

## References

1. Berg W, Schanz H, Eisenwinter B, Schorch P. Häufigkeitsverteilung und Trendentwicklung von Harnsteinsubstanzen. *Urologe [A]* 1992; 31:98–102.
2. Luque de Castro MD. Determination of oxalic acid in urine: a review. *J Pharm Biomed Anal* 1988; 6/1:1–13.

3. Zerwekh JE, Drake E, Gregory J, Griffith D, Hofmann AF, Menon M, Pak CYC. Assay of urinary oxalate: six methodologies compared. *Clin Chem* 1983; 29/11:1977–80.
4. Hodgkinson A, Williams A. An improved colorimetric procedure for urine oxalate. *Clin Chim Acta* 1972; 36:127–32.
5. Berg W, Gutsche B, Schäfer F, Beck G. Eine modifizierte Methode zur quantitativen Oxalsäurebestimmung im Harn. *Z Urol Nephrol* 1979; 72:323–30.
6. Zarembski PM, Hodgkinson A. The fluorimetric determination of oxalate in blood and other biological fluids. *Biochem J* 1965; 96:717–21.
7. Koehl C, Abeassis I. Determination of oxalic acid in urine by atomic absorption spectrophotometry. *Clin Chim Acta* 1976; 70:71–7.
8. Hesse A. Oxalsäure und Urolithiasis. Darmstadt: GIT Verlag Ernst Giebeler, 1983:3–29.
9. Wolthers BG, Hayer M. The determination of oxalic acid in plasma and urine by means of capillary gas chromatography. *Clin Chim Acta* 1982; 120:87–102.
10. Duggan DE, Walker RW, Noll RM, van den Heuvel WJA. Determination of urinary oxalic acid and of oxalate pool size by stable isotope dilution. *Anal Biochem* 1979; 94:477–82.
11. Koolstra W, Wolthers BG, Hayer M, Elzinga H. Development of a reference method for determining urinary oxalate by means of isotope dilution-mass-spectrometry (ID – MS) and its use in testing existing assays for urinary oxalate. *Clin Chim Acta* 1987; 170:227–36.
12. Hallson PC, Rose GA. A simplified and rapid enzymatic method for determination of urinary oxalate. *Clin Chim Acta* 1974; 55:29–39.
13. Classen A, Hesse A. Measurement of urinary oxalate: an enzymatic and an ion chromatographic method compared. *J Clin Chem Clin Biochem* 1987; 25:95–9.
14. Costello I, Hatch M, Bourke E. An enzymatic method for the spectrophotometric determination of oxalic acid. *J Lab Clin Med* 1976; 87:903–8.
15. Kohlbecker G, Richter L, Butz M. Determination of oxalate in urine using oxalate oxidase: comparison with oxalate decarboxylase. *J Clin Chem Clin Biochem* 1989; 17:309–13.
16. Kok WT, Groenendijk G, Brinkmann UA, Frei RW. Determination of oxalic acid in biological matrices by liquid chromatography with amperometric detection. *J Chromatogr* 1984; 315:271–8.
17. Schwille PO, Manoharan M, Rübenapf G, Wölfel G, Berens H. Oxalate measurement in the picomol range by ion chromatography: values in fasting plasma and urine of controls and patients with idiopathic calcium urolithiasis. *J Clin Chem Clin Biochem* 1989; 27:87–96.
18. Petrarulo M, Bianco O, Marangella M. Ion chromatographic determination of plasma oxalate in healthy subjects, in patients with chronic renal failure and in cases of hyperoxaluric syndromes. *J Chromatogr* 1990; 511:223–31.
19. Millan A, Grases JM, Grases F. Rapid determination of urinary oxalate by high-performance liquid chromatography. *J Chromatogr* 1990; 529:402–7.
20. Frey IDR, Starkey BJ. The determination of oxalate in urine and plasma by high-performance liquid chromatography. *Ann Clin Biochem* 1991; 28:581–7.
21. Brega A, Quadri A, Villa P, Prandini P, Wei J, Lucarelli C. Improved high-performance liquid chromatographic determination of plasma and urine oxalate in the clinical diagnostic laboratory. *J Liq Chromatogr* 1992; 15/3:501–11.
22. Toyoda M. Simple ion-chromatographic method for determination of urinary oxalate. *Urol Res* 1966; 13:179–83.
23. Wissenschaftliche Tabellen Geigy, Teilband Körperflüssigkeiten, 8. Auflage. Basel, 1977:51.
24. Meldrum WB, Newlin IG. Solubility of benzidine sulfate and benzidine hydrochloride in hydrochloric acid solutions. *Industrial and Engineering Chemistry*, October 1929.
25. Mazzachi BC, Teubner JK, Ryall RL. Factors affecting measurement of urinary oxalate. *Clin Chem* 1984; 30/8:1339–43.
26. Petrarulo M, Marangella M, Bianco O, Marchesini A, Linari F. Preventing ascorbate interference in ion chromatographic determination of urinary oxalate: four methods compared. *Clin Chem* 1990; 36/9:1642–5.
27. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedure for method comparison studies in clinical chemistry, Part I. *J Clin Chem Clin Biochem* 1983; 21:709–20.
28. Passing H, Bablok W. Comparison of several regression procedures for method comparison studies and determination of sample size. Application of linear regression procedure for method comparison studies in clinical chemistry, Part II. *J Clin Chem Clin Biochem* 1984; 22:431–45.

*Received December 2, 1996/February 6, 1997*

Corresponding author: Prof. Dr. A. Hesse, Experimentelle Urologie, Klinik und Poliklinik für Urologie, Universität Bonn, Sigmund-Freud-Straße 25, D-53105 Bonn, Germany